

Pythium attrantheridium sp. nov.: taxonomy and comparison with related species

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Pythium attrantheridium sp. nov. is a new species isolated from cavity spot lesions of carrots as well as apple and cherry seedlings from various locations widely distributed in Canada and the USA. This fungus is closely related to the heterothallic *P. intermedium*, but is distinguished by: (1) unique molecular characteristics; (2) unique morphological characteristics; and (3) mating incompatibility with *P. intermedium*. The ITS region of the nuclear rDNA of all strains of *P. attrantheridium* studied is different from that of all other known *Pythium* spp. The oogonia attract a large number of antheridia when compatible mating types contact each other. The positive mating type produces zoospores unlike those of *P. intermedium*. Thus, biological, morphological and molecular data support the recognition of a new species.

INTRODUCTION

Members of the genus *Pythium* are ubiquitous and occupy several ecological niches (van der Plaats-Niterink 1981). These organisms belong to the family Pythiaceae, phylum *Heterokonta*, kingdom *Straminipila* (Dick 2001b). More than 120 species of the genus have been described, and the most comprehensive taxonomic account with descriptions of species is provided by van der Plaats-Niterink (1981), whose key was updated by Dick (1990). The taxonomic classification is primarily based on the comparison of morphological characteristics, including calculations of proportions of certain structures (Dick 1990). While most *Pythium* spp. are saprophytes in various types of soils and aquatic environments, many others are important plant pathogens. The most common diseases caused by species belonging to this genus are damping-off of seedlings and root rot. *Pythium* species have been also isolated from mosquito larvae (Hafidh & Kelly 1982) and one can infect mammals (Mendoza, Kaufman & Standard 1987).

Morphology-based taxonomy is increasingly being supplemented by molecular characteristics of a given

species. The ITS region of the rDNA has become a useful tool in fungal taxonomy and can be used to identify or detect different *Pythium* spp. (e.g. Chen 1992, Chen, Hoy & Schneider 1992, Lévesque, Harlton & de Cock 1998, Paul 2001, Paul 2003). The ITS sequence of four isolates from cavity spots on carrots (Allain-Boulé *et al.* 2004) was identical to that of *P. intermedium* isolates from Washington state (Mazzola *et al.* 2001) and to that of some of the isolates recovered from cherry roots (Packer & Clay 2000) that were recently sequenced. Mazzola *et al.* (2001, 2002) reported some sequence differences between the *P. intermedium* recovered from apple and reference strains of *P. intermedium*. The differences between the apple isolates and reference strains were minor compared to differences between the apple isolates *Pythium* MM1 (*P. aff. macrosporum*), *Pythium* MM2 (*P. aff. iwayamae*), *Pythium* MM3 (*P. aff. oedochilum*), *Pythium* MM4 (*P. aff. rostratum*) and *Pythium* MM5 (*P. aff. vexans*) of this study that were immediately flagged as possible five new species (Mazzola, Reganold & Lévesque 2002). Because *P. intermedium* is heterothallic and so likely to have more genetic variation than homothallic species, the differences observed between the apple isolates and the reference strains of *P. intermedium* were attributed to intraspecific variation.

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In this study, we compared seven strains of the material from carrot, apple and wild cherry from Québec City (Québec), Wenatchee (Washington State) and Bloomington (Indiana), with reference strains of *P. intermedium*, the closest species by phylogenetic and morphological analysis. Morphology, biological compatibility and molecular data revealed that the carrot, apple and wild cherry isolates represented a new species from *P. intermedium* and other known species of *Pythium*.

MATERIALS AND METHODS

Fungal isolates

Pythium spp. (DAOM 230383, DAOM 230384, DAOM 230385) were isolated from commercially grown carrot roots with cavity spot lesions collected from fields near Québec City (Québec). The isolation procedure followed the method described by Benard & Punja (1995). Pieces of tissue 2 mm² were cut from the edges of cavity spot lesions, plated on corn meal agar (CMA) containing pimarin (10 mg l⁻¹), vancomycin (200 mg l⁻¹), streptomycin (50 mg l⁻¹), penicillin (250 mg l⁻¹), Pentachloronitrobenzene (100 mg l⁻¹) and rose bengal (22 mg l⁻¹) and incubated at room temperature (20 °C). Subcultures were conserved on CMA under mineral oil (Sigma, St Louis, MO) (van der Plaats-Niterink 1981) at room temperature and at 10 °. Strains from Mazzola *et al.* (2001) previously identified as *P. intermedium* (DAOM 230389, DAOM 230387, DAOM 230388) and a strain from Packer & Clay (2000) (DAOM 230386) were also used. Strains from Mark Mazzola, Wenatchee (WA) were isolated from apple orchard soils, and the strain from Alissa Packer, Bloomington (IN) was isolated from wild cherry seedlings.

Sequencing rDNA

Extraction of DNA was made following modified Lyse-n-Gotm (Pierce, Rockford, IL, 1999) protocol. Mycelium was put in 10 µl of Lyse-n-Gotm buffer and incubated twice under the following conditions: 2 min at 95 ° and 2 min at 4 °.

DNA was amplified by PCR in a total volume of 20 µl. Primers at 0.08 µM concentration of Un-Up 18S42 (CGTAACAAGGTTTCCGTAGGTGAAC) and Py-Lo 28S22 (GTTTCTTTTCCTCCGCTTATTAATATG) were used with the Taq Titanium (BD Bioscience Clontech, Palo Alto, CA) polymerase (1×) and 0.1 mM of dNTP's. PCR tubes were placed in the Genius thermal cycler (Techne, Princeton, NJ) and processed with the following temperature profile: 1 cycle 3 min at 95 °; 40 cycles 45 s at 95 °, 45 s at 68 °, 1 min 30 at 72 °; with a final extension of 8 min at 72 °. Visual quantification was made by comparison to a DNA low mass ladder (Invitrogen, Carlsbad, CA) following electrophoresis on a 1% agarose gel stained with ethidium bromide.

Sequence reactions were made following Big Dye Terminator (BDT) version 2 (Applied Biosystems, Foster City, CA) protocol with four primers to get complete ITS 1 and ITS 2 sequences forward and reverse: Un-Up 18S42, Oom-Up 5.8S55 (TGCGA-TACGTAATGCGAATT) and Py-Lo 28S22, Oom-Lo 5.8S47 (ATTACGTATCGCAGTTTCGCAG). The final volume of 20 µl comprised 3 µl of (5×) BDT Seq Buffer, 2 µl of BDT Sequence mix version 2, between 2 and 10 ng of DNA template obtained by PCR and a final concentration of 0.16 µM of the primer. Amplified sequenced products were purified by ethanol 95% precipitation and resuspended in formamide.

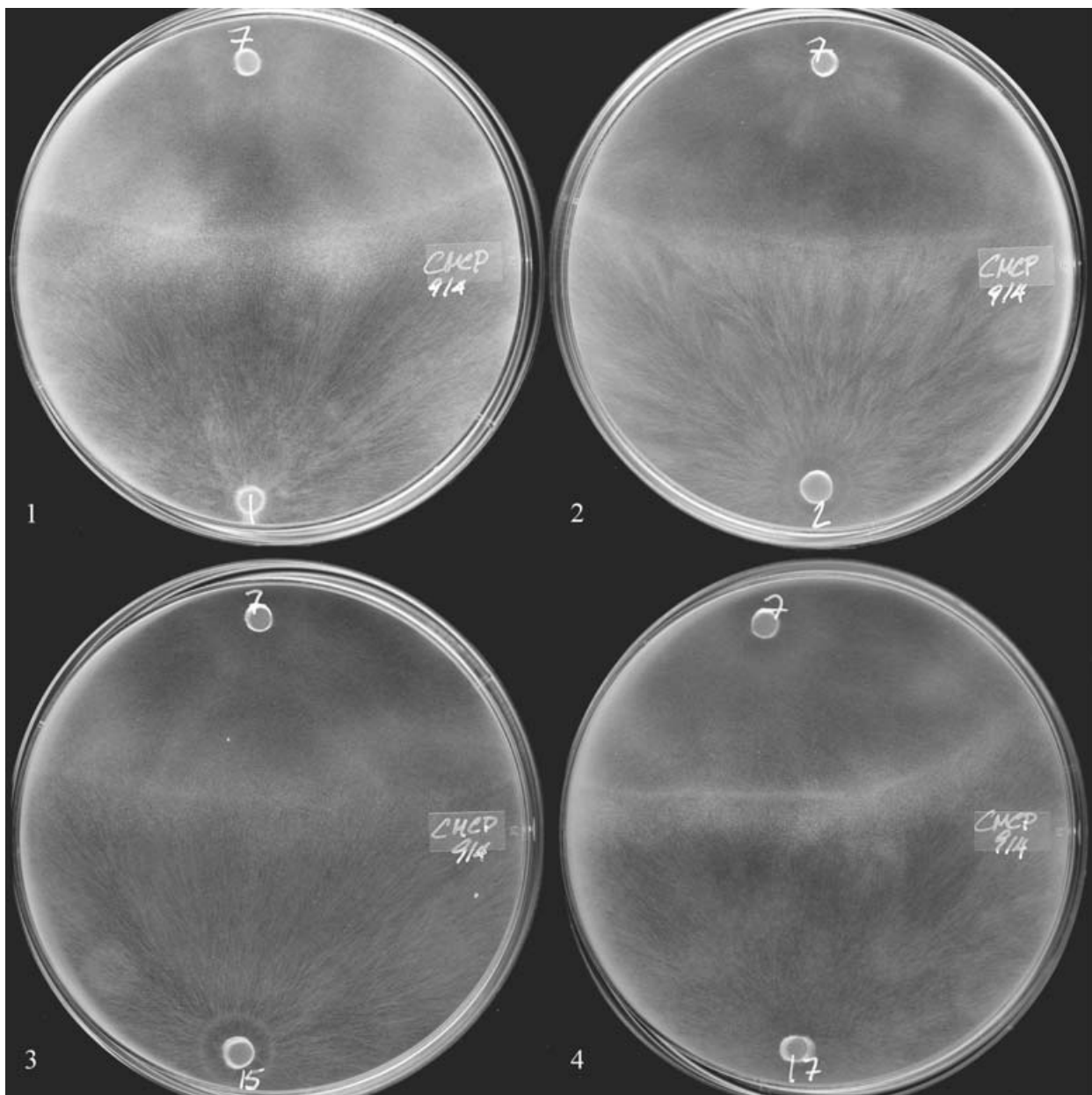
The tubes were transferred into an ABI Prism Genetic Analyzer (model 310; Applied Biosystems) for electrophoresis and analysis. Sequences were edited using SeqMan II (version 5.00, DNASTar, 2001). A BLAST search was performed on GenBank to find closely related species for analysis. A more complete unpublished database did not yield more closely related species than the BLAST on GenBank (C.A.L., data not shown). The ITS sequence alignments done by Megalign (DNASTar) was edited manually before the parsimony phylogenetic analysis which was performed with PAUP version 4.062 software (Swofford 2001).

Mating compatibility

As *Pythium intermedium* is heterothallic, strains of the putative new species were tested against the two mating types (+, -) of *P. intermedium* in three sets of mating comparisons. In the first set, all different strains of the new *Pythium* were placed between *P. intermedium* CBS 221.68 (+) and *P. intermedium* CBS 266.38 (-) on CMA, in a Petri dish separated into three parts. In the second set, DAOM 238303 (-) of the new *Pythium* was chosen arbitrarily to be in contact with all other strains, to find the mating type of each strain. In the third step, all strains were placed between DAOM 230389 (+) and DAOM 230383 (-), two compatible strains, to confirm the mating compatibility of the strains under test. All mating experiments were made on 1:1 CMA and potato-carrot agar (PCA; van der Plaats-Niterink 1981) at room temperature (*ca* 20 °). Positive (+) types were designated following observation of oogonia and negative types (-) on the presence of antheridia as confirmed microscopically.

Morphological comparisons

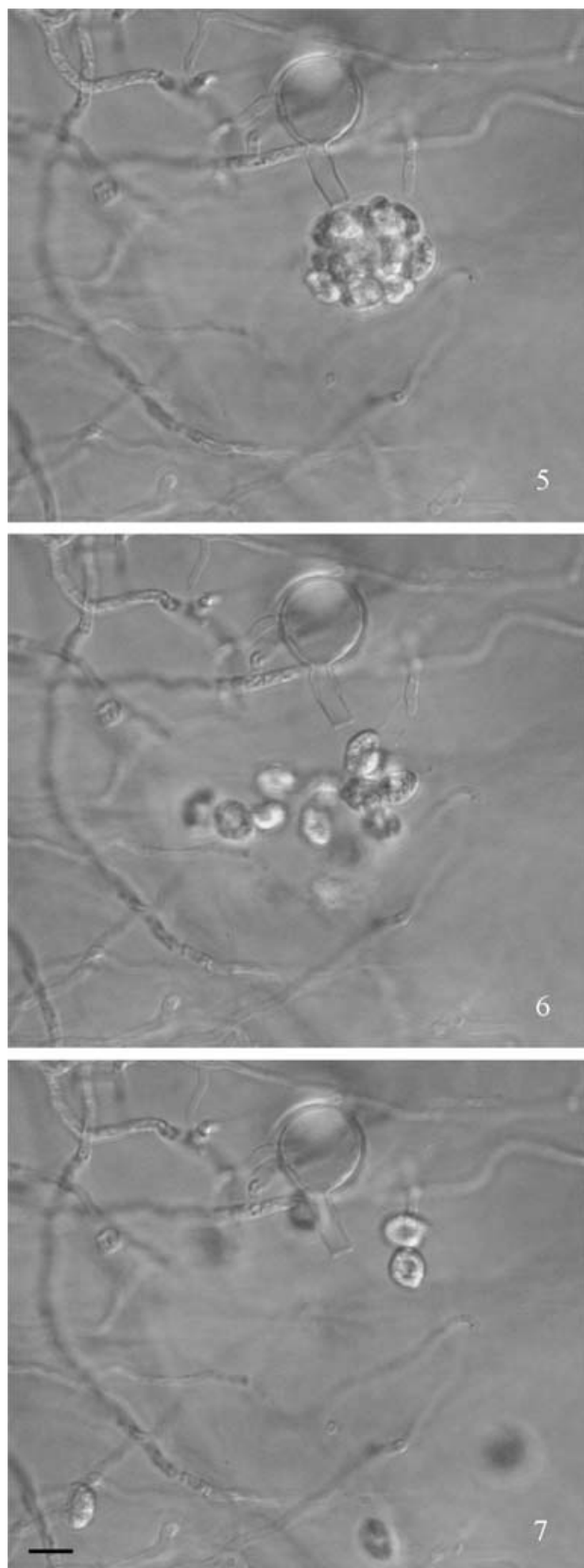
Production of oospores of *Pythium intermedium* was enhanced on a 1:1 medium of PCA and CMA (van der Plaats-Niterink 1981) and was used to observe oospores of the new *Pythium*. Small agar fragments from the heterothallic contact line were mounted in water on glass slides and observed under a Nikon (Tokyo) Differential Interference Contrast (DIC)



Figs 1–4. Contact line of positive heterothallism reactions of positive (+) mating type DAOM 230389 (no. 7 on the Petri dish) from Washington on 1:1 carrot-potato and cornmeal agar. **Fig. 1.** Heterothallic reaction with (–) DAOM 230383 (no. 1) from Québec province. **Fig. 2.** Heterothallic reaction with (–) DAOM 230384 (no. 2) from Québec. **Fig. 3.** Heterothallic reaction with (–) DAOM 230388 (no. 15) from Washington state. **Fig. 4.** Heterothallic reaction with (–) DAOM 230386 (no. 17) from Indiana. Note the difference between growth pattern of mating type: (+) DAOM 230389 radiate pattern and (–) chrysanthemum pattern. Petri dish = 9 cm diam.

Eclipse 800 microscope. Digital camera images were captured with Nikon ACT-1 v 2.10 software. Images of the morphology of different strains were used to produce scale drawings and measurements of the different structures. Oogonia, oospores, ooplasts, hyphal swellings, sporangia and zoospores were measured to the nearest 0.1 µm on a Macintosh computer using the public domain National Institute of Health Image program, version 1.62 (available on the Internet at <http://rsb.info.nih.gov/nih-image/>). Measurements of 20 oospores in oogonia were taken to calculate wall index, aplerotic index, and ooplast index as described by Dick (1990).

Sporangia and zoospores were produced using the method of van der Plaats-Niterink (1981). Autoclaved grass blades were left on a culture surface for 2 d and then placed in autoclaved pond water. Sporangia and free zoospores were present after 24 h. Growth rate was evaluated at 5°, 10°, 15°, 20°, 25° and 30° during 5 d on CMA. Subcultures of the seven strains of the new *Pythium* sp. were prepared at room temperature (ca 20°) where they were left overnight for initial growth out of the 2 mm² plug. Radial measurements were made with a Digimatic electronic caliper (Mitutoyo Corporation, Kanagawa).



Figs 5–7. Three steps of zoospore discharge process from the sporangia of *Pythium attrantheridium* (DAOM 230389) (+). Bar = 10 μ m.

RESULTS

Mating experiments

Mating tests confirm that all the strains of the novel *Pythium* sp. are incompatible with each of the two mating strains of *P. intermedium* studied by van der Plaats-Niterink (1981). These assays show that the mycelium is either growing between the mycelium of *P. intermedium* or avoiding it completely by leaving a line free of any mycelium at the interface. Through the mating tests, DAOM 230389 was found to be compatible with all other strains of the new taxon. A well-marked heterothallic contact line that produced numerous oospores was observed when compatible mating types were placed together. (Fig. 1) DAOM 230389 (+) produces oogonia in the presence of a compatible (–) strain, and is defined as the positive mating type (+). All other selected strains are of the (–) mating type and produce antheridia.

TAXONOMY

Pythium attrantheridium Allain-Boulé & Lévesque, sp. nov. (Figs 2–49)

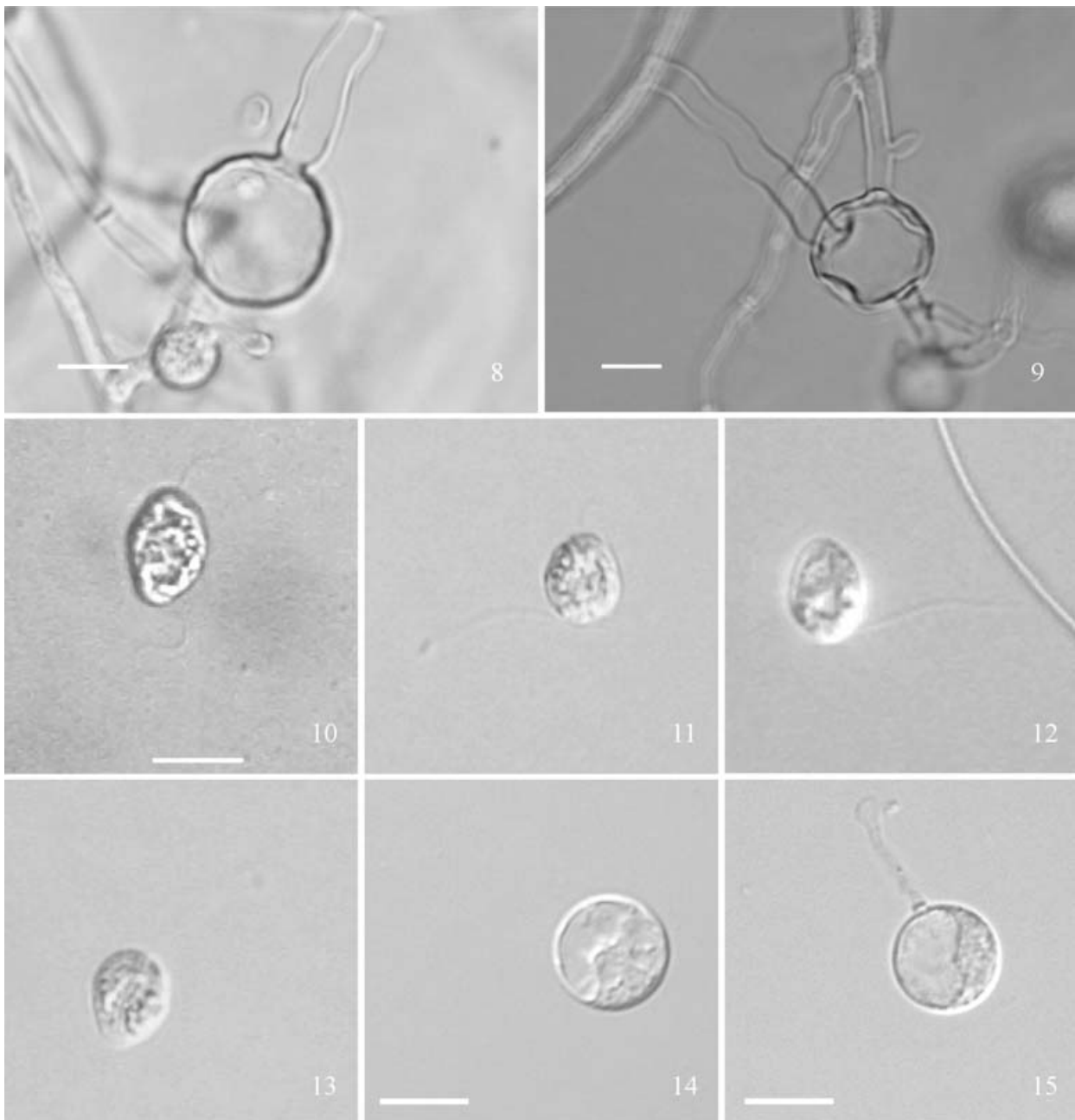
Etym.: Contracted from *attraho* (Latin), to attract, and antheridium.

Thalli dioecii; (–) antheridiferi; (+) oosporiferi, zoosporangiferi. Species haec ab *Pythium intermedium* differt coloniis rosulatis (–) vel actiniformibus (+), incrementis diurnis tardis (18 mm –) (11 mm +), hyphis angustis, oogoniis terminalibus magnis, antheridiis ephemeris magnis multicellularis, appressorii fabiformibus vel allan. Zoosporangia (+) globosa, terminalia vel intercalaria, (9.5–) 13–19 (–23), in medio 16.5 μ m, zoosporis 3–15. Zoosporae 20° in 2 diebus formantes. Tubi evacuationis, (13–) 27–55.5 \times (3–) 4–5 (–7.5) in medio 27 \times 5 μ m. E *Pythio intermedio* segregatum, non interfertile. E *Pythio* speciebus cognito genetice distinctum in partes ITS (Fig. 50).

Typus: Specimen siccum DAOM 233296 (DAOM 230389 \times 230383 in cultura duali).

Specimens mated: **USA**: *Washington*: Chelan: Wenatchee (47° 24' N, 120° 12' W), isolated from orchard soil baited with *Malus* sp., 18 Aug 1998, M. Mazzola MAZ_d2a [Lévesque 2180] DAOM 230389 (+) strain (oogonial), – singly and mated with a (–) strain (antheridial) (cultura viva in CCFC). – **Canada**: *Québec*: Île d'Orléans: St-Laurent de l'Île d'Orléans (46° 52' N, 71° 01' W), isolated from *Daucus carota* in storage, Jan. 2001, N. Allain-Boulé [Lévesque 3031] DAOM 230383 (cultura viva in CCFC).

Colonies on CMA, PCA and 1:1 CMA:PCA are submerged. Positive mating type DAOM 230389 (+) generates a vague radiate pattern, and the negative mating type a clear chrysanthemum-like pattern (with acute triangles) on corn meal agar (Figs 1–4). Thin aerial hyphae are present on potato-dextrose agar (PDA). The main hyphae are 3.5 μ m wide on average, but can be up to 5 μ m wide and as narrow as 2 μ m. The mycelium is hyaline, yellowish, and well-branched (Fig. 16). Hyphae growing in spirals can often be



Figs 8–15. *Pythium attrantheridium* (DAOM 230389) (+). **Figs 8–9.** Empty sporangium after release of zoospores; note the variation in the discharge tube length. **Figs 10–13.** Swimming zoospores. **Figs 14–15.** Encysted zoospores. **Fig. 15.** Encysted zoospore germinating. Bars = 10 μ m.

observed in and on the medium. Septa are lacking except in old, mostly empty hyphae, or where they delimit reproductive organs. *Hyphal swellings* are abundant, rarely catenulate (Fig. 18) and their spherical shape can be easily mistaken for an oogonium. Those spherical swelling structures are (19–) 24 (–28) av. 24.5 μ m wide which is very similar to oogonia. They can be terminal, subterminal or intercalary. As well, they become separated from the hypha by a septum and can regerminate and create another mycelium when the right humid conditions are present. Appressoria in bean chain shape are especially abundant in the presence of a sexually compatible strain, but can be also observed in pure

culture in bean (Fig. 33) and sausage shape (Figs 17 and 34).

Sporangia and *zoospores* are produced by the positive mating strain DAOM 230389 (+), but not by any of the negative type strains. *Sporangia* are terminal or intercalary, globose, and (9.5–) 13–19 (–23) av. 16.5 μ m in size. *Zoospores* are formed at room temperature (ca 20 °) in less than 24 h. Sporangia contain 3–15 zoospores. Discharge tubes are about (13–) 27 (–55.5) av. 27 μ m in length and (3–) 4–5 (7.5) av. 5 μ m in width (Figs 5–7, 8–9).

Oogonia are produced in dual culture of two compatible isolates, forming a whitish line of contact,

Table 1. Morphological differences between *Pythium intermedium* and *P. attrantheridium*.

Morphological differences	<i>P. intermedium</i> ^a	<i>P. attrantheridium</i>
Colony pattern	Vaguely radiate	(–) Chrysanthemum, (+) radiate
Sporangia	Not produced ^b	Terminal or intercalary
Hyphal swellings	Often catenulate, to 25 µm	Extremely rarely catenulate, to 30 µm
Appressoria	Not mentioned	In bean and sausage shape
Oogonia	Intercalary (predominant) ^c or terminal, (18–) 19–22 (–27) (av. 21.5) µm	Terminal, 15– (22–25) –30 (av. 23) µm
Antheridia	Persistent, branched often bifurcate near the oogonium, cells 1–7, crook-necked	Soon vanishing after fertilization, numerous, entirely cover oogonium, not bifurcate, cells 1–20 +
Oospore	(13–) 16–20 (–21) (av. 17.5) µm, wall 1–2 µm thick, sometimes 2 oospores in an oogonium	13– (19–20) –27 (av. 19) µm, walls (1–) 1.5 µm thick. Sometimes 2 ooplasts in an oospore
Cardinal temperature	Min. 5 °, opt 20–25 °, max. 30 °, 30 mm d ^{–1} , faster radial growth	Variable: (–) min. below 5 °, opt 22 °, max. 30 °, 18 mm d ^{–1} . (+) min. 5 °, opt 25 °, max. 30 °, 11 mm d ^{–1}
Indices	Aplerotic index <0.60 ^c , wall index and ooplast index are not available	Oospore wall index is 0.36; applerotic index is 0.60; and ooplast index is 0.09

^a According to the description of van der Plaats-Niterink (1981), except when mentioned.

^b In van der Plaats Niterink (1981) and Dick (1990).

^c In Dick (1990).

Table 2. Major sequence differences between *Pythium intermedium* and *P. attrantheridium*, their position base on *P. attrantheridium* sequence, their type and their nature.

Intron	Type of difference	Position	<i>P. intermedium</i>	<i>P. attrantheridium</i>
ITS 1	Deletion	13–14, 77–78, 81–82	A, GTAT, TAC	–
ITS 1	Transition	32, 72, 106	A, C, A	G, T, G
ITS 1	Transversion	81, 168	A, C	T, A
ITS 2	Deletion	470–471	C	–
ITS 2	Transition	89, 94–95, 392, 396, 400, 411, 446	C, AT, T, T, C, C, T	T, GC, C, C, T, T, C
ITS 2	Transversion	70, 79, 85, 196, 360, 374, 399, 403, 445, 455, 460	G, T, G, T, G, G, T, A, G, G, G	T, G, A, A, C, C, A, T, T, C, C
ITS 2	Insertion	378–379, 412–413, 466, 467	–	GA, GT, T, G

rather sharp to the side of the oogonial isolate. Oogonia are terminal and (15–) 22–25 (–30) av. 23 µm diam. The thickness of the oogonial walls is approximately 0.5 µm. *Antheridia* are declinous with a rather long, branched stalk that vanishes soon after fertilization. Antheridia are inflated and have a broad apical attachment with oogonia. Numerous antheridial cells contact individual oogonia. *Oospores* are plerotic or applerotic, (13–) 19–20 (–27) av. 19 µm diam, with walls (1–) 1.5 av. 1.5 µm thick. Sometimes there are two ooplasts in an oospore. The wall index is 0.36; the applerotic index is 0.60; and the ooplast index is 0.09 (see Dick 1990 for definitions of terms).

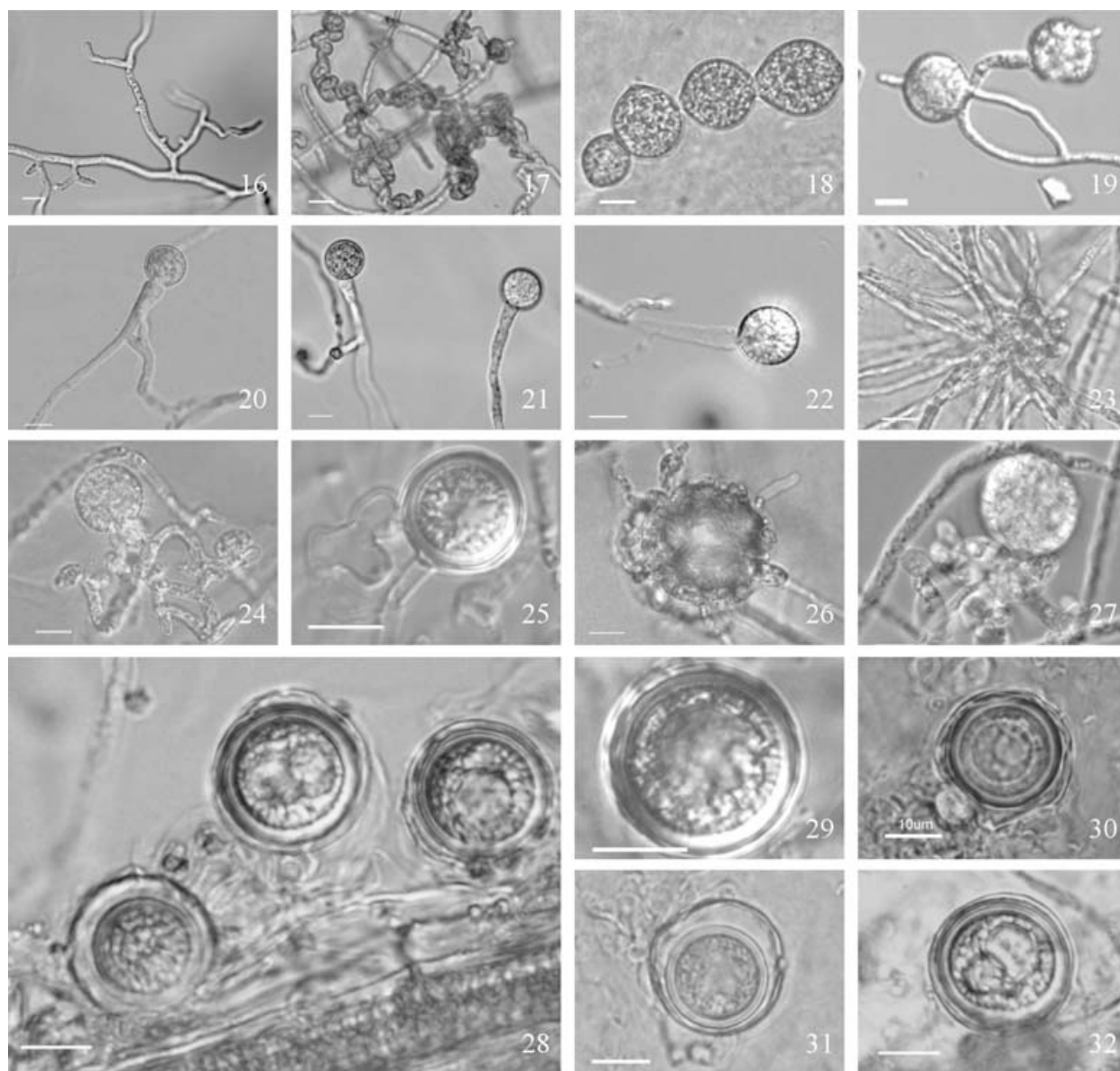
Cardinal temperatures vary depending on mating types and their origin. Mating type (–): minimum below 5 °, optimum 22 °, maximum 30 °. The daily growth rate at 25 ° on CMA is about 18 mm d^{–1}. Mating type (+) DAOM 230389 exhibits a slower growth rate: minimum 5 °, optimum 25 °, maximum over 30 °, and the daily growth rate at 25 ° on corn meal agar is about 11 mm d^{–1}.

Other cultures studied: **Canada:** Québec: Île-d'Orléans County: St-Laurent-de-l'Île-d'Orléans, 46° 52' N 71° 01' W, on cavity spot lesion of *Daucus carota*, Jan. 2001, *N. A.-Boulé* (DAOM 230384 (–), DAOM 230385 (–)). – **USA:** Indiana: Monroe County: Bloomington,

39° 8' N, 86° 37' W, isolated from cherry tree seedlings, Nov. 2001, *A. Paker* (DAOM 230386 (–)). *Washington:* Chelan County: Wenatchee, 47° 24' N 120° 12' W, on apple soil, Aug. 1998, *M. Mazzola* (DAOM 230387 (–), DAOM 230388 (–)).

ITS sequence analysis

The ITS region (ITS 1, 5.8S, ITS 2) of the new *Pythium* sequence was 863 bp in length (GenBank accession no. AY286014). The ITS 1, 5.8S and ITS 2 are 219, 159, and 485 bp in length, respectively. There were 35 major differences between the new *Pythium* and *P. intermedium* in the ITS 1 and ITS 2 (Table 2). *P. intermedium* was the closest genetic neighbour of *P. attrantheridium* in a BLAST search of GenBank, and when compared to a database of all (neo-)type strains (Lévesque, unpubl.). The phylogenetic tree shows that the distance between *P. intermedium* and the new *Pythium* is small, but comparable to the distance between, for example, *P. irregulare* and *P. paroecandrum* (Fig. 33). A high bootstrap value between *P. intermedium* and the new *Pythium* shows that the separation is well-supported by the data. The phylogenetic cluster made by *P. intermedium* and the new *Pythium* is isolated from any other species.



Figs 16–32. *Pythium attrantheridium*. **Fig. 16.** Mycelium, (DAOM 230383) (–). **Fig. 17.** Appressoria, sausage shape (DAOM 230387) (–). **Fig. 18.** Catenulate spherical hyphal swellings, extremely rare (DAOM 230384) (–). **Figs 19–22.** Spherical hyphal swellings are abundant and could be confused with oogonia (DAOM 230383) (–). **Fig. 23.** Radiate multiple antheridia growing towards an oogonium, (DAOM 230383) (–) antheridia and (DAOM 230389) (+) oogonium under antheridia in the middle. **Figs 24–27.** Oogonium fertilized by one or many antheridia. **Figs 24–25.** Holotype (+, –), i.e. dual culture of DAOM 230383 (–) and DAOM 230389 (+). **Fig. 26.** DAOM 230386 (–) and DAOM 230389 (+). **Fig. 27.** DAOM 230385 (–) and DAOM 230389 (+). **Figs 28–29.** Type (+, –), i.e. DAOM 230383 (–) and DAOM 230389 (+). **Fig. 28.** Three types of oospores, plerotic and applerotic with one or two ooplasts (rare), 40 d after fertilization. **Fig. 29.** Aplerotic oospore with one ooplast. **Fig. 30.** Aplerotic oospore with 2 ooplasts (DAOM 230386 (–) and DAOM 230389 (+)). **Fig. 31.** Plerotic oospore with one ooplast (DAOM 230388 (–) and DAOM 230389 (+)). **Fig. 32.** Aplerotic oospore with two ooplasts, holotype (+, –), i.e. DAOM 230383 (–) and DAOM 230389 (+). Bar = 10 µm.

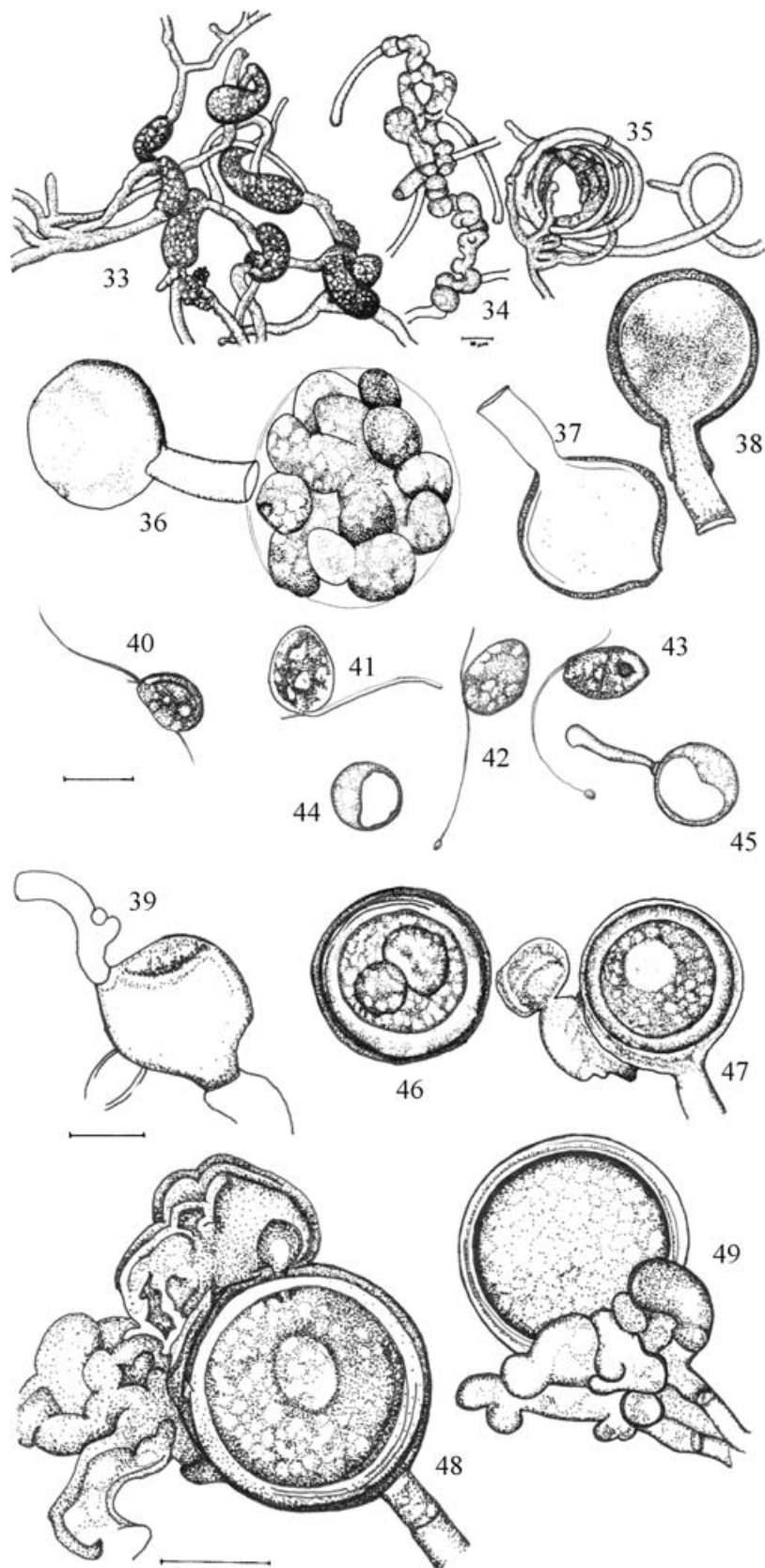
DISCUSSION

Isolates of *Pythium attrantheridium* have been isolated from cavity spot symptoms on carrot roots in Québec (Allain-Boulé *et al.* 2004), cherry tree seedlings in Indiana (Packer & Clay 2000), and in soil of apple tree fields in Washington state (Mazzola 1999, Mazzola, Reganold & Lévesque 2002). Mazzola (1999) concluded that plant pathogenic fungi in the genera *Phytophthora*, *Pythium*, and *Rhizoctonia*, represented an increasing

proportion of the fungal community isolated from seedling roots as orchard age increased.

P. attrantheridium was found in a study which concluded that native pathogens influence cherry tree distribution by preventing the growth of seedlings around mature trees of the same species (Packer & Clay 2000).[†] This is somewhat similar to the apple replant problem

[†] See Mycological Research News (*Mycological Research* **104** (7): 770, July 2000).



Figs 33–49. *Pythium attrantheridium*. **Fig. 33.** Reniform appressoria (DAOM 230387 (–)). **Fig. 34.** Allantoid appressoria (DAOM 230387 (–)). **Fig. 35.** Spiral hyphae (DAOM 230386 (–)). **Figs 36–45.** DAOM 230389 (+). **Fig. 36.** Sporangium, discharge tube and wide ready to release zoospores. **Figs 37–39.** Empty sporangia. **Figs 40–43.** Swimming zoospores. **Fig. 44.** Encysted zoospore. **Fig. 45.** Encysted zoospore germinating. **Fig. 46.** Oospore with two ooplasts in an oogonium, holotype (+, –). **Fig. 47.** Oospore in an oogonium. **Fig. 48.** Upper focus of an oospore in an oogonia, holotype (+, –). **Fig. 49.** Oogonium being fertilized. Bars = 10 µm.

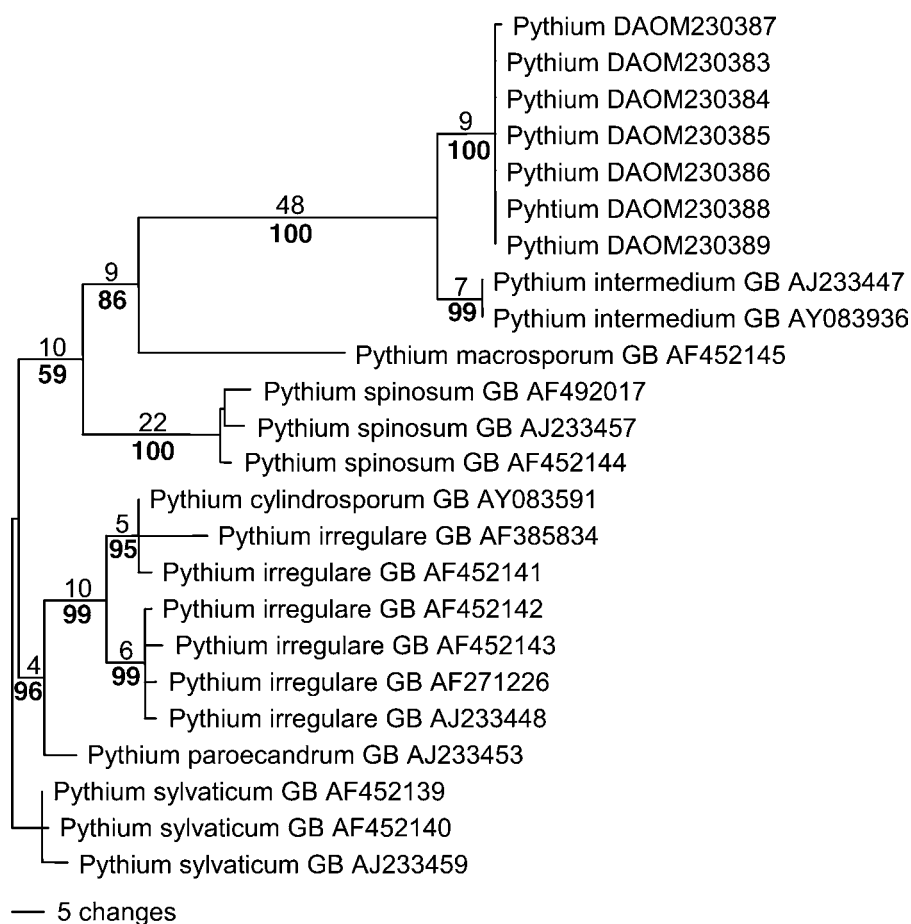


Fig. 50. One of the four equally parsimonious trees resulting from a heuristic search performed on the ITS rDNA dataset. Bootstrap values (1000 reps) are shown at branches. Length 211, CI=0.83, RI=0.95. All taxa except *Pythium attrantheridium* are from GenBank.

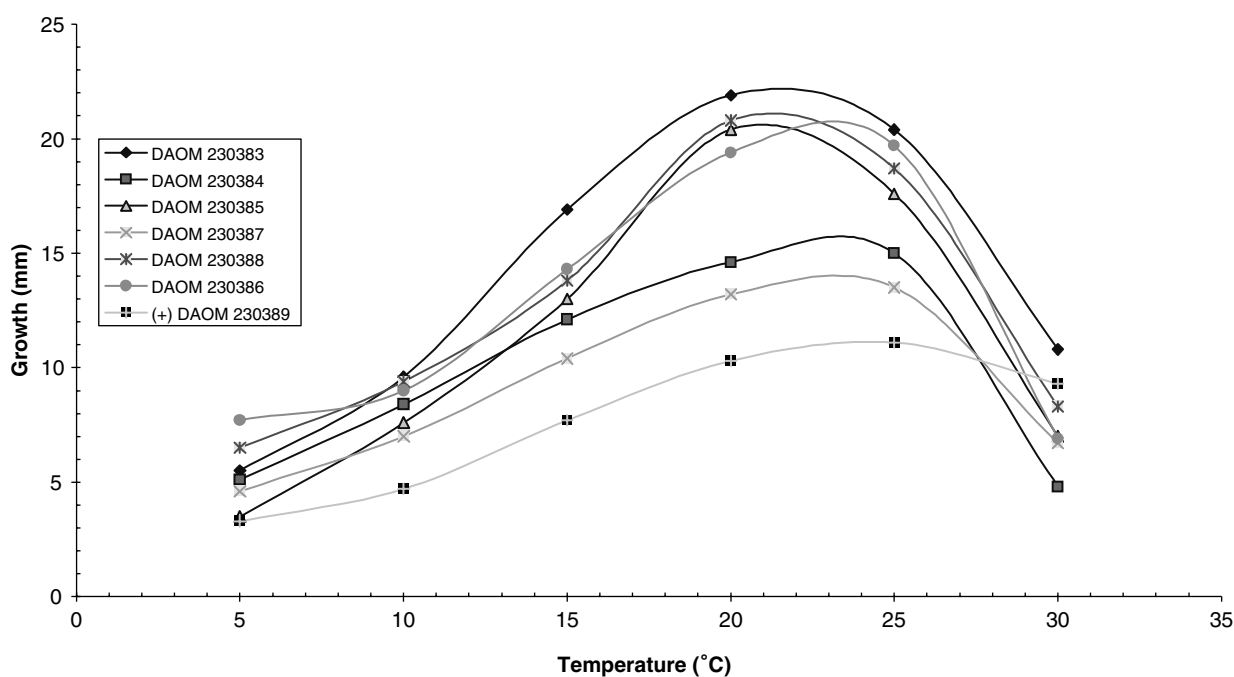


Fig. 51. Growth of *Pythium attrantheridium* on corn meal agar after two days at different temperatures. Positive mating type (DAOM 230389 (+)) tolerates higher temperature and grows slower than negative mating types.

where the microbiota associated with old orchard trees prevents the subsequent establishment of young trees. *P. attrantheridium* could be an important component of this phenomenon (Mazzola, Reganold & Lévesque 2002), and could play an ecological role in the tree distribution. It could play a similar ecological role in a biennial plant, colonizing the tap root in the first year and preventing germination of seeds around it in the second year. This is a novel ecological role for *Pythium* spp., which merits further exploration as to the relationship between colonization and pathogenicity by *P. attrantheridium* in perennial root systems, and the potential to incite damping off and seedling mortality for the same plant species. The damping-off potential of *P. attrantheridium* on seedlings remains to be demonstrated.

The incompatibility between *P. attrantheridium* and *P. intermedium* is a crucial element in the segregation of the new species. The incompatibility with its closest genetic relative shows that this new taxon is a biological species in the traditional sense.

Morphological characters were markedly different between *P. attrantheridium* and closely related species. First, the main hyphae of *P. attrantheridium* are narrow (av. 3.5 (–5) μm) compared to *Pythium* in general (5–7 (–10) μm ; van der Plaats-Niterink 1981). *P. attrantheridium* (–) produced clear chrysanthemum-like colonies on PCA, whereas *P. intermedium* produced vague radiate patterns (Table 2). The growth of *P. attrantheridium* was much slower (11–18 mm d^{–1}; Fig. 34) than that of *P. intermedium* (30 mm d^{–1}). At the microscopic level, *P. attrantheridium* produced several types of appressoria, as described by van der Plaats-Niterink (1981); some were kidney bean or sausage-shaped, arranged somewhat like beads on a chain. *P. attrantheridium* rarely showed catenulate hyphal swelling. The oogonia and oospores of *P. attrantheridium* were larger (av. +1.5 μm) than those of *P. intermedium*, and the oogonia of the new species were contacted by many more antheridia during fertilization than is typical for other *Pythium* species. Oospores could contain two ooplasts. While *P. intermedium* can produce two oospores in an oogonium, this was not observed in *P. attrantheridium*. When compared with other closely related species, *P. attrantheridium* had thinner oospore walls and grew more slowly than *P. macrosporum*; antheridia were never monoclinal as in *P. paroecandrum* or *P. irregulare*; oogonia did not have finger-like ornamentations as in *P. spinosum* or *P. irregulare*; and the colonies did not produce cottony aerial mycelium on corn meal agar as is seen in *P. sylvaticum*.

P. attrantheridium produces zoospores in culture, unlike *P. intermedium* as described by de Bary (1881), and more recently by van der Plaats-Niterink (1981) and Dick (1990). However, Atkinson (1895) described *P. intermedium* (as *Artotrogus intermedius*) as producing zoospores. The development of the sporangia was very similar to that of *P. attrantheridium*, except that some zoospores were fusoid instead of oval. In addition,

Atkinson described how a zoospore reaches an amoeboid movement and ‘tends to cut the organism into two parts ... and progress until complete fission results in the formation of two zoospores which are oval in form with the cilium attached directly at the smaller end’ (Atkinson 1895). No double zoospores were observed in *P. attrantheridium*.

Zoospores of *P. attrantheridium* have two swimming patterns: (1) turning around its flagella while moving forward; and (2) pulsing while moving forward in a circle or a regular 8-pattern in water free of obstacles. When zoospores encountered obstacles, the movement became more chaotic as the zoospore moved along them. Dick (2001a) mentioned that contraction of the flagella could be a major characteristic for species identification, but he also noted that this feature had never been reported previously. Closer observations are needed to confirm that the pulsation of the zoospore is created by the contraction of the flagella.

P. attrantheridium was clearly differentiated from *P. intermedium* based on morphology, mating incompatibility and molecular data. The fact that it was found in an undisturbed environment of North America as well as in agriculture soils indicates that this species may be native to North America.

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